

Title of the Invention

SOLUBLE GLcNAC PHOSPHOTRANSFERASE

BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention relates to a soluble GlcNAc phosphotransferase, a method of making the same and a method of phosphorylating with the same.

10 Discussion of the Background

Lysosomes are organelles in eukaryotic cells that function in the degradation of macromolecules into component parts that can be reused in biosynthetic pathways or discharged by the cell as waste. Normally, these macromolecules are broken down by enzymes known as lysosomal enzymes or lysosomal hydrolases. However, when a lysosomal enzyme is not present in the lysosome or does not function properly, the enzymes specific macromolecular substrate accumulates in the lysosome as "storage material" causing a variety of diseases, collectively known as lysosomal storage diseases.

Lysosomal storage diseases can cause chronic illness and death in hundreds of individuals each year. There are approximately 50 known lysosomal storage diseases, e.g., Pompe Disease, Hurler Syndrome, Fabry Disease, Maroteaux-Lamy Syndrome (mucopolysaccharidosis VI), Morquio Syndrome (mucopolysaccharidosis IV), Hunter Syndrome (mucopolysaccharidosis II), Farber Disease, Acid Lipase Deficiency, Krabbe Disease, and Sly Syndrome (mucopolysaccharidosis VII). In each of these diseases,

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lysosomes are unable to degrade a specific compound or group of compounds because the enzyme that catalyzes a specific degradation reaction is missing from the lysosome, is present in low concentrations in the lysosome, or is present at sufficient concentrations in the lysosome but is not functioning properly.

5           Lysosomal storage diseases have been studied extensively and the enzymes (or lack thereof) responsible for particular diseases have been identified. Most of the diseases are caused by a deficiency of the appropriate enzyme in the lysosome, often due to mutations or deletions in the structural gene for the enzyme. For some lysosomal storage diseases, the enzyme deficiency is caused by the inability of the cell to target and  
10       transport the enzymes to the lysosome, e.g., I-cell disease and pseudo-Hurler polydystrophy.

          Lysosomal Storage diseases have been studied extensively and the enzymes (or lack thereof) responsible for particular diseases have been identified (Scriver, Beaudet, Sly, and Vale, eds., *The Metabolic Basis of Inherited Disease*, 6th Edition, 1989,  
15       Lysosomal Enzymes, Part 11, Chapters 61-72, pp. 1565-1839). Within each disease, the severity and the age at which the disease presents may be a function of the amount of residual lysosomal enzyme that exists in the patient.

          The lysosomal targeting pathways have been studied extensively and the process by which lysosomal enzymes are synthesized and transported to the lysosome has been  
20       well described. Kornfeld, S. (1986). "Trafficking of lysosomal enzymes in normal and disease states." *Journal of Clinical Investigation* 77: 1-6 and Kornfeld, S. (1990). "Lysosomal enzyme targeting." *Biochem. Soc. Trans.* 18: 367-374. Generally, lysosomal enzymes are synthesized by membrane-bound polysomes in the rough

endoplasmic reticulum ("RER") along with secretory glycoproteins. In the RER, lysosomal enzymes acquire N-linked oligosaccharides by the en-bloc transfer of a preformed oligosaccharide from dolichol phosphate containing 2 N-acetylglucosamine, 9-mannose and 3-glucose. Glycosylated lysosomal enzymes are then transported to the Golgi apparatus along with secretory proteins. In the cis-Golgi or intermediate compartment lysosomal enzymes are specifically and uniquely modified by the transfer of GlcNAc-phosphate to specific mannoses. In a second step, the GlcNAc is removed thereby exposing the mannose 6-phosphate ("M6P") targeting determinant. The lysosomal enzymes with the exposed M6P binds to M6P receptors in the trans-Golgi and is transported to the endosome and then to the lysosome. In the lysosome, the phosphates are rapidly removed by lysosomal phosphatases and the mannoses are removed by lysosomal mannosidases (Einstein, R. and Gabel, C.A. (1991). "Cell- and ligand-specific dephosphorylation of acid hydrolases: evidence that the mannose 6-phosphate is controlled by compartmentalization." *Journal of Cell Biology* 112: 81-94).

The synthesis of lysosomal enzymes having exposed M6P is catalyzed by two different enzymes, both of which are essential if the synthesis is to occur. The first enzyme is UDP-N-acetylglucosamine: lysosomal enzyme N-Acetylglucosamine-1-phosphotransferase ("GlcNAc-phosphotransferase") (E.C. 2.7.8.17). GlcNAc-phosphotransferase catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-GlcNAc to the 6 position of 1,2-linked mannoses on the lysosomal enzyme. The recognition and addition of N-acetylglucosamine-1-phosphate to lysosomal hydrolases by GlcNAc-phosphotransferase is the critical and determining step in lysosomal targeting. The second step is catalyzed by N-acetylglucosamine-1-phosphodiesterase -N-

Acetylglucosaminidase ("phosphodiester  $\alpha$ -GlcNAcase") (E.C. 3.1.4.45).

Phosphodiester  $\alpha$ -GlcNAcase catalyzes the removal of N-Acetylglucosamine from the GlcNAc-phosphate modified lysosomal enzyme to generate a terminal M6P on the lysosomal enzyme.

40 5 GlcNAc-phosphotransferase is an enzyme that contains <sup>S</sup> of six subunit;  $\alpha\beta\gamma_2$ .

411603 40 The  $\alpha$  and  $\beta$  subunits are encoded on a single mRNA and proteolytically cleaved after translation. The  $\gamma$  subunit is encoded on a separate mRNA molecule. Removal of the transmembrane domain from the  $\alpha/\beta$  polyprotein results in a soluble form of the enzyme.

10023888-122101 10 This soluble form of GlcNAc-phosphotransferase facilitates a quicker and simpler purification scheme that reduces or eliminates the need for detergents to extract the non-soluble GlcNAc-phosphotransferase from membrane fractions. However, notwithstanding the ease of purification the recombinant soluble GlcNAc-phosphotransferase was not efficiently subject to post-translational proteolytic cleavage when expressed in mammalian cells such as 293T cells and CHO-K1 cells. Uncleaved 15 forms of  $\alpha/\beta/\gamma$  GlcNAc-phosphotransferase had poor GlcNAc phosphotransferase activity.

To solve this problem, the present inventors have discovered that by interposing a unique proteolytic cleavage site between the  $\alpha$  and  $\beta$  subunits in the GlcNAc polyprotein, the polyprotein is cleaved and when expressed with the  $\gamma$ -subunit effectively 20 phosphorylates an enzyme substrate.

In addition, the present inventor has discovered, quite unexpectedly, that the  $\alpha$  and  $\beta$  subunits alone is catalytically active. Furthermore, the absence of the  $\gamma$ -subunit results in loss of substrate specificity to only those lysosomal enzymes targeted via the

mannose-6-phosphate targeting systems, e.g., acid  $\alpha$ -glucosidase, acid  $\beta$ -galactosidase,  $\beta$ -hexaminidase, and others as described herein. This loss of substrate specificity allows the soluble GlcNAc-phosphotransferase containing the  $\alpha$  and  $\beta$  tetramer to effectively phosphorylated any glycoprotein having an appropriate acceptor oligosaccharide, for example, mannose-6 through mannose-9 isomers (Baranski et al (1990) Cell 63:281-291).

### SUMMARY OF THE INVENTION

Thus, an object of the present invention is a method of phosphorylating a protein comprising contacting the protein with a soluble GlcNAc-phosphotransferase.

10 In one embodiment, the protein comprises an asparagine-linked oligosaccharide with a high mannose structure.

In another embodiment the soluble GlcNAc-phosphotransferase contains an  $\alpha$  subunit, a  $\beta$  subunit and a proteolytic cleavage site interposed between said  $\alpha$  and  $\beta$  subunits, wherein said proteolytic cleavage site is not natural to said GlcNAc-phosphotrasferase.

15 In another embodiment, the soluble GlcNAc-phosphotransferase comprises  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits.

It is another object of the present invention that in the method of phosphorylating a protein in a host cell, which contains an isolated polynucleotide encoding the soluble GlcNAc-phosphotransferase. The method is accomplished by culturing the host cell for a time under conditions suitable for expression of the soluble GlcNAc-phosphotransferase; the soluble GlcNAc-phosphotransferase is isolated and then may be employed to phosphorylate glycoproteins.

It is another object of the invention that in a method of phosphorylating a protein, a host cell containing an isolated polynucleotide encoding soluble GlcNAc-phosphotransferase is cultured for a time under conditions suitable for expression of the soluble GlcNAc-phosphotransferase, where the soluble GlcNAc-phosphotransferase has an  $\alpha$  subunit, a  $\beta$  subunit, and a proteolytic cleavage site interposed between the  $\alpha$  and  $\beta$  subunits, where the proteolytic cleavage site is not endogenous to the GlcNAc-phosphotransferase; the soluble GlcNAc-phosphotransferase is isolated, the isolated GlcNAc-phosphotransferase is cleaved with a proteolytic enzyme specific for the proteolytic cleavage site; and the  $\alpha$  and  $\beta$  subunits are expressed with a  $\gamma$  subunit of GlcNAc-phosphotransferase to effectuate phosphorylation.

It is another object of the invention to provide soluble GlcNAc-phosphotransferase containing an  $\alpha$  subunit, a  $\beta$  subunit and a proteolytic cleavage site interposed between the  $\alpha$  and  $\beta$  subunits, where the proteolytic cleavage site is not endogenous to GlcNAc-phosphotransferase; as well as polynucleotides which encode the soluble GlcNAc-phosphotransferase.

It is another object of the invention to provide methods of producing an  $\alpha$  and  $\beta$  GlcNAc-phosphotransferase polypeptide by culturing a host cell of the invention (that which contains and expresses the GlcNAc-phosphotransferase) for a time and under conditions suitable for expression of the  $\alpha$  and  $\beta$  GlcNAc-phosphotransferase polypeptide and collecting the protein produced. In one embodiment, the  $\alpha$  and  $\beta$  GlcNAc-phosphotransferase subunits are cleaved in the host cell by a protease which is expressed in the cell, wherein the protease is specific for the proteolytic cleavage site interposed between the  $\alpha$  and  $\beta$  subunits or are cleaved after collection from the host.

It is another object of the invention to provide a method for making a GlcNAc-phosphotransferase by combining the  $\alpha$  and  $\beta$  subunits of GlcNAc-phosphotransferase with a  $\gamma$  subunit of GlcNAc-phosphotransferase and the GlcNAc-phosphotransferase obtained by such methods. Such methods may be accomplished *in vitro* by combining  
5 already expressed and collected protein subunits or by introducing a polynucleotide which encodes a  $\gamma$  subunit of GlcNAc-phosphotransferase into a host cell which also has the  $\alpha$  and  $\beta$  subunits being expressed; culturing the host cell for a time and under conditions for the expression of the various subunits; and collecting the GlcNAc-phosphotransferase .

10 It is another object of the invention to provide methods of treating a patient suffering from a lysosomal storage disease, by preparing a phosphorylated lysosomal hydrolase, employing the advantages of the soluble GlcNAc-phosphotransferase, and subsequently contacting the lysosomal hydrolase with an isolated GlcNAc-phosphotransferase to produce a phosphorylated lysosomal hydrolase; the modified  
15 protein is then administered to a patient in need of treatment.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Model structure of GlcNAc-phosphotransferase.

Figure 2. Schematic diagram of the phosphorylation of a lysosomal enzyme.

20 Figure 3. Schematic diagram of the reaction of GlcNAc-phosphotransferase. One unit of GlcNAc-phosphotransferase activity is defined as 1 pmol of GlcNAc phosphate transferred to  $\alpha$ -methylmannoside per hour in a reaction containing 150  $\mu$ M UDP-GlcNAc and 100 mM  $\alpha$ -methylmannoside at 37°C.

Figure 4. Molecular engineering of GlcNAc phosphotransferase. cDNA encoding recombinant soluble human GlcNAc-phosphotransferase  $\alpha/\beta$  subunits was made by replacing N-terminal and C-terminal putative transmembrane domains with a secretion signal (Ig) and an epitope tag (HPC4) and stop codon, respectively.

Figure 5. Schematic diagram for introducing proteolytic cleavage sites between the alpha and beta subunits. The junction between the alpha and beta subunits is shown (SEQ ID NO:26) and various cleavage sites are shown below (SEQ ID NOS:22-25) the junction of the cleavage sites and  $\alpha/\beta$  sequence is shown in SEQ ID NOS:33,35, 37 and 38.

Figure 6. (A) Schematic diagram for engineering GlcNAc-phosphotransferase. PCR products were prepared to replace the sequence between *Hpa* I to *Xba* I to introduce unique restriction enzyme sites around the  $\alpha/\beta$  cleavage site. (B) Schematic and nucleotide sequence of PCR primer sites and target nucleotides. The amino acid sequence at the top of the Figure is SEQ ID NO:26 and the DNA sequence below is SEQ ID NO:27. Primers 2 and 3 have the sequences of SEQ ID NOS:28 and 29, respectively. The amino acid sequences at the bottom of the Figure are SEQ ID NOS:30 and 31 and the nucleotide sequence (+ strand) is SEQ ID NO:32 and the complement of SEQ ID NO:32. (C). Overview of the introduction of protease cleavage sites into the engineered GlcNAc-phosphotransferase. The sequences at the top of the Figure correspond to SEQ ID NOS:28-31 and fragments of SEQ ID NO:32 and its complement. The amino acid sequence and corresponding DNA sequence of the Factor Xa cleavage site is SEQ ID NOS: 33 and 34, respectively. The amino acid sequence and corresponding DNA sequence of the Furin cleavage site is SEQ ID NOS:35 and 36, respectively. The amino



acid sequence of the Enterokinase cleavage site is SEQ ID NO:37. The amino acid sequence of the Genease I cleavage site is in SEQ ID NO:38 and the wildtype amino acid sequence shown in SEQ ID NO:26. (amino acids 8-33).

Figure 7. Schematic overview of the expression of  $\alpha/\beta/\gamma$  GlcNAc phosphotransferase on a single mRNA molecule and translated employing an Internal Ribosome Entry Sequence (IRES).

#### DETAILED DESCRIPTION OF THE INVENTION

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, New York (2001), Current Protocols in Molecular Biology, Ausubel et al (eds.), John Wiley & Sons, New York (2001) and the various references cited therein.

Within the context of the present invention "Isolated" means separated out of its natural environment.

Within the context of the present invention "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

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The term "GlcNAc-phosphotransferase" as used herein refers to enzymes that are  
5 capable of catalyzing the transfer of N-acetylglucosamine-1-phosphate from UDP-GlcNAc to the 6' position of 1,2-linked mannoses on lysosomal enzymes. The GlcNAc-phosphotrasferase is composed of six subunits: 2  $\alpha$  subunits, 2  $\beta$ -subunits and 2  $\gamma$  subunits. The amino acid sequence of the  $\alpha$  subunit is shown in SEQ ID NO:4 (amino acids 1-928), the human  $\beta$  subunit is shown in SEQ ID NO:5 (amino acids 1-328), and  
10 the human  $\gamma$  subunit is shown in SEQ ID NO:7 (amino acids 25-305, signal sequence is in amino acids 1-24).

A novel soluble GlcNAc phosphotransferase has been prepared which is composed of a non-endogenous proteolytic cleavage site interposed between the  $\alpha$  and  $\beta$  subunits. When combined with the  $\gamma$  subunit, this GlcNAc phosphotransferase exhibits  
15 high levels of activity. The soluble GlcNAc-phosphotransferase protein or polypeptide as used herein is understood to mean the sequences exemplified in this application as well as those which have substantial identity to SEQ ID NO:2. This soluble GlcNAc-phosphotransferase is missing the transmembrane domain of the non-engineered GlcNAc-phosphotransferase and has a Furin proteolytic cleavage site interposed between  
20 the  $\alpha$  and  $\beta$  subunits.

The partial rat and *Drosophila melanogaster*  $\alpha/\beta$  GlcNAc-phosphotransferase amino acid sequences are shown in SEQ ID NO: 14 and 16, respectively.

Preferably, the GlcNAc-phosphotransferase polypeptides are those which are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to the GlcNAc-phosphotransferase amino acid sequences described herein.

Polynucleotides which encode the  $\alpha$  and  $\beta$  subunits of GlcNAc-phosphotransferase or soluble GlcNAc-phosphotransferase mean the sequences exemplified in this application as well as those which have substantial identity to those sequences and which encode an enzyme having the activity of the  $\alpha$  and  $\beta$  subunits of GlcNAc-phosphotransferase. Preferably, such polynucleotides are those which hybridize under stringent conditions and are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to those sequences

The nucleotide sequence for the human  $\alpha/\beta$  subunit precursor cDNA is shown in SEQ ID NO:3 (nucleotides 165-3932), the nucleotide sequence of the  $\alpha$  subunit is in nucleotides 165-2948 of SEQ ID NO:3, the nucleotide sequence of the  $\beta$  subunit is shown in nucleotides 2949-3932 of SEQ ID NO:3, and the nucleotide sequence of the  $\gamma$  subunit is shown in SEQ ID NO:6 (nucleotides 25-305). The soluble GlcNAc-phosphotransferase nucleotide sequence is shown in SEQ ID NO:1. The partial rat and Drosophila melanogaster  $\alpha/\beta$  GlcNAc-phosphotransferase nucleotide sequences are shown in SEQ ID NO: 13 and 15, respectively.

The term "phosphodiester  $\alpha$  -GlcNAcase" as used herein refers to enzymes that are capable of catalyzing the removal of N-Acetylglucosamine from GlcNAc-phosphate-mannose diester modified lysosomal enzymes to generate terminal M6P.

Polynucleotides which encode phosphodiester  $\alpha$  -GlcNAcase as used herein is understood to mean the sequences exemplified in this application as well as those which

have substantial identity to SEQ ID NO:19 (murine) or SEQ ID NO:17 (human) and which encode an enzyme having the activity of phosphodiester  $\alpha$  -GlcNAcase.

Preferably, such polynucleotides are those which hybridize under stringent conditions and are at least 70%, preferably at least 80% and more preferably at least 90% to 95%

5 identical to SEQ ID NOS:17 and/or 19.

The phosphodiester  $\alpha$  -GlcNAcase protein or polypeptide as used herein is understood to mean the sequences exemplified in this application as well as those which have substantial identity to SEQ ID NO:20 (murine) or SEQ ID NO:18 (human).

10 Preferably, such polypeptides are those which are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to SEQ ID NOS:18 and/or 20.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a polynucleotide will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in  
15 different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing).

20 Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50

nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1X to 2X SSC (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA--DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984):  $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$ ; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe.  $T_m$  is reduced by about 1°C. for each 1% of mismatching; thus,  $T_m$ , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the  $T_m$  can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point ( $T_m$ ) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3,

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or 4°C. lower than the thermal melting point ( $T_m$ ); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C. lower than the thermal melting point ( $T_m$ ); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C. lower than the thermal melting point ( $T_m$ ). Using the equation, 5 hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a  $T_m$  of less than 45°C. (aqueous solution) or 32°C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the 10 hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

15 Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the BestFit or Gap pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). BestFit uses the local homology algorithm of Smith and Waterman, Advances in Applied 20 Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. Gap performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as BestFit, to determine the

degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as BestFit to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as blosum45 or blosum80, may be selected to optimize identity, similarity or homology scores.

Recombinant expression vectors containing a nucleic acid sequence encoding the enzymes can be prepared using well known techniques. The expression vectors include a DNA sequence operably linked to suitable transcriptional or translational regulatory nucleotide sequences such as those derived from mammalian, microbial, viral, or insect genes. Examples of regulatory sequences include transcriptional promoters, operators, enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the DNA sequence for the appropriate enzyme. Thus, a promoter nucleotide sequence is operably linked to a GlcNAc-phosphotransferase DNA sequence if the promoter nucleotide sequence controls the transcription of the appropriate DNA sequence.

The ability to replicate in the desired host cells, usually conferred by an origin of replication and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not naturally associated with GlcNAc-phosphotransferase can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in-

frame to the enzyme sequence so that the enzyme is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the appropriate polypeptide. The signal peptide may be cleaved from the polypeptide upon secretion of enzyme from the cell.

5           Suitable host cells for expression of the GlcNAc-phosphotransferase include prokaryotes, yeast, archae, and other eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are well known in the art, *e.g.*, Pouwels *et al.* Cloning Vectors: A Laboratory Manual, Elsevier, New York (1985). The vector may be a plasmid vector, a single or double-  
10           stranded phage vector, or a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsulated virus by well-known techniques for infection and transduction. Viral  
15           vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells. Cell-free translation systems could also be employed to produce the enzymes using RNAs derived from the present DNA constructs.

20           Prokaryotes useful as host cells in the present invention include gram negative or gram positive organisms such as *E. coli* or *Bacilli*. In a prokaryotic host cell, a polypeptide may include a N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant GlcNAc-phosphotransferase or phosphodiester



-GlcNAcase polypeptide. Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include -lactamase and the lactose promoter system.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. To construct an expression vector using pBR322, an appropriate promoter and a DNA sequence are inserted into the pBR322 vector.

Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, Wisconsin, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include  $\beta$ -lactamase (penicillinase), lactose promoter system (Chang *et al.*, Nature 275:615, (1978); and Goeddel *et al.*, Nature 281:544, (1979)), tryptophan (trp) promoter system (Goeddel *et al.*, Nucl. Acids Res. 8:4057, (1980)), and tac promoter (Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, p. 412 (1982)).

Yeasts useful as host cells in the present invention include those from the genus *Saccharomyces*, *Pichia*, *K. Actinomycetes* and *Kluyveromyces*. Yeast vectors will often

contain an origin of replication sequence from a 2 $\mu$  yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-

- 5 phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem. 255:2073, (1980)) or other glycolytic enzymes (Holland *et al.*, Biochem. 17:4900, (1978)) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.
- 10 Other suitable vectors and promoters for use in yeast expression are further described in Fleer *et al.*, Gene, 107:285-195 (1991). Other suitable promoters and vectors for yeast and yeast transformation protocols are well known in the art.

- Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen *et al.*, *Proceedings of the National Academy of Sciences*
- 15 *USA*, 75:1929 (1978). The Hinnen protocol selects for Trp.sup.+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10  $\mu$ g/ml adenine, and 20  $\mu$ g/ml uracil.

- Mammalian or insect host cell culture systems well known in the art could also be employed to express recombinant GlcNAc-phosphotransferase or phosphodiester -
- 20 GlcNAcase polypeptides, *e.g.*, Baculovirus systems for production of heterologous proteins in insect cells (Luckow and Summers, Bio/Technology 6:47 (1988)) or Chinese hamster ovary (CHO) cells for mammalian expression may be used. Transcriptional and translational control sequences for mammalian host cell expression vectors may be

excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell, *e.g.*, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication. Exemplary expression vectors for use in mammalian host cells are well known in the art.

The enzymes of the present invention may, when beneficial, be expressed as a fusion protein that has the enzyme attached to a fusion segment. The fusion segment often aids in protein purification, *e.g.*, by permitting the fusion protein to be isolated and purified by affinity chromatography. Fusion proteins can be produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of the enzyme. Preferred fusion segments include, but are not limited to, glutathione-S-transferase,  $\beta$ -galactosidase, a poly-histidine segment capable of binding to a divalent metal ion, and maltose binding protein. In addition, the HPC-4 epitope purification system may be employed to facilitate purification of the enzymes of the present invention. The HPC-4 system is described in U.S. Patent No. 5,202,253, the relevant disclosure of which is herein incorporated by reference.

According to the present invention, isolated and purified GlcNAc-phosphotransferase enzymes may be produced by the recombinant expression systems

described above. The method comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes the enzyme under conditions sufficient to promote expression of the enzyme. The enzyme is then recovered from culture medium or cell extracts, depending upon the expression system employed. As is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is secreted into the culture medium. When expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, *e.g.*, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Also, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Further, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media (*e.g.*, silica gel having pendant methyl or other aliphatic groups) can be employed to further purify the enzyme. Some or all of the foregoing purification steps, in various combinations, are well known in the art and can be employed to provide an isolated and purified recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or

more concentration, salting-out, ion exchange, affinity purification, or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

5           The GlcNAc phosphotransferase  $\alpha$  and  $\beta$  subunits are encoded by a single mRNA. The natural GlcNAc-phosphotransferase consists of a transmembrane domain and a proteolytic cleavage site, which is employed during post-translational processing to separate the  $\alpha$  and  $\beta$  polypeptides. Genetic manipulation of the GlcNAc-phosphotransferase coding sequence to remove the transmembrane domain yields a  $\alpha$  and  
10    $\beta$  polyprotein, which is soluble and more easily recoverable when the coding sequence is expressed in a culture system.

          The soluble GlcNAc-phosphotransferase  $\alpha$  and  $\beta$  polyprotein can be further engineered to remove the endogenous or natural proteolytic cleavage site. As used herein “endogenous or natural proteolytic cleavage site” means the cleavage site, which is  
15   found in the naturally occurring wildtype GlcNAc-phosphotransferase  $\alpha$  and  $\beta$  protein, and which is encoded from the corresponding gene or nucleotide sequence. Preferably, following removal or concurrent with the removal the proteolytic cleavage site is replaced with a non-natural or non-endogenous proteolytic cleavage site is inserted between the  $\alpha$  and  $\beta$  subunits. This proteolytic site should catalyze the cleavage of a  
20   peptide bond in a site determinative manner. Examples of such non-natural proteolytic cleavage sites include Furin, Factor Xa, Enterokinase, and Genease (SEQ ID NOS:22-25).

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The soluble GlcNAc-phosphotransferase engineered to contain a non-natural proteolytic site can then be subjected to the corresponding protease to cleave the  $\alpha\beta$  polyprotein at the proteolytic site yielding separate  $\alpha$  and  $\beta$  GlcNAc-phosphotransferase subunits. The GlcNAc-phosphotransferase  $\alpha$  and  $\beta$  polyprotein can be cleaved either *in vitro* or *in vivo*. *In vitro* proteolysis includes containing either crude, preferably partially purified, and more preferably purified, GlcNAc-phosphotransferase polyprotein and then subjecting the GlcNAc phosphotransferase polyprotein to proteolysis reactions, these reactions conditions will vary depending on the enzyme used and concentration of the protein and proteolytic enzyme.

10 *In vivo* proteolysis includes overexpressing the gene encoding the protease with the soluble GlcNAc-phosphotransferase in a cell and then isolating the separated  $\alpha$  and  $\beta$  subunits. The coexpression can be performed by cotransfection of the genes, transfection of the soluble GlcNAc-phosphotransferase polynucleotide followed by the transfection of the protease, transfection of the soluble GlcNAc phosphotransferase into a cell that stably expresses the protease or transfecting the protease into a cell, which stably expresses the soluble GlcNAc-phosphotransferase. Alternatively, the soluble GlcNAc-phosphotransferase can be expressed in a cell, which has a natural or endogenous protease present in the cell, and use that protease to achieve proteolytic cleavage.

15 The invention also provides methods of phosphorylating a protein with the soluble GlcNAc-phosphotransferase  $\alpha$  and  $\beta$  subunits alone or in combination with the  $\gamma$  subunit. Additionally, the phosphorylated proteins may subsequently be treated with phosphodiester  $\alpha$ -GlcNAcase. The GlcNAc-phosphotransferase soluble or non-soluble, containing only the  $\alpha$  and  $\beta$  subunits or all of  $\alpha$ ,  $\beta$  and  $\gamma$  can be assayed for activity in the

same manner. For example, the GlcNAc-phosphotransferase can be measured by assessing the ability to transfer GlcNAc phosphate to  $\alpha$ -methylmannosidase per hour in a reaction containing UDP-GlcNAc and  $\alpha$ -methylmannoside.

5 In one embodiment of the present invention, the phosphorylated proteins are lysosomal enzymes that utilize the M6P transport system and thus obtain phosphorylated lysosomal enzymes. Lysosomal hydrolases are produced by treating the high mannose hydrolases with GlcNAc-phosphotransferase which catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-GlcNAc to the 6' position of 1,2-linked or  
10 other mannoses on the hydrolase.

Examples of such lysosomal enzymes (and diseases linked to their deficiency) include  $\alpha$ -glucosidase (Pompe Disease),  $\alpha$ -L-iduronidase (Hurler Syndrome),  $\alpha$ -galactosidase A (Fabry Disease), arylsulfatase (Maroteaux-Lamy Syndrome), N-acetylgalactosamine-6-sulfatase or  $\beta$ -galactosidase (Morquio Syndrome), iduronate 2-  
15 sulfatase (Hunter Syndrome), ceramidase (Farber Disease), galactocerebrosidase (Krabbe Disease),  $\beta$ -glucuronidase (Sly Syndrome), Heparan N-sulfatase (Sanfilippo A), N-Acetyl- $\alpha$ -glucosaminidase (Sanfilippo B), Acetyl CoA- $\alpha$ -glucosaminide N-acetyl transferase, N-acetyl-glucosamine-6 sulfatase (Sanfilippo D), Galactose 6-sulfatase (Morquio A), Arylsulfatase A, B, and C (Multiple Sulfatase Deficiency), Arylsulfatase A  
20 Cerebroside (Metachromatic Leukodystrophy), Ganglioside (Mucopolipidosis IV), Acid  $\beta$ -galactosidase  $G_{M1}$  Ganglioside ( $G_{M1}$  Gangliosidosis), Acid  $\beta$ -galactosidase (Galactosialidosis), Hexosaminidase A (Tay-Sachs and Variants), Hexosaminidase B (Sandhoff),  $\alpha$ -fucosidase (Fucosidosis),  $\alpha$ -N-Acetyl galactosaminidase (Schindler

Disease), Glycoprotein Neuraminidase (Sialidosis), Aspartylglucosamine amidase (Aspartylglucosaminuria), Acid Lipase (Wolman Disease), Acid Ceramidase (Farber Lipogranulomatosis), Lysosomal Sphingomyelinase and other Sphingomyelinase (Nieman-Pick).

5 In another embodiment of the invention, the lysosomal hydrolase glucocerebrosidase whose deficiency is the causative agent of Gaucher's disease may be subject to phosphorylation with the soluble GlcNAc phosphotransferase  $\alpha$  and  $\beta$  subunits alone. This modified GBA may then be treated with phosphodiester  $\alpha$ -GlcNAcase to complete the modification of the GBA thereby making the enzyme available for targeting  
10 tissues via the M6P receptor. This modified GBA has been found to bind to the mannose receptor with high affinity resulting in an increased bioavailability of the enzyme compared to the current GBA employed in therapeutic protocols, particularly in lung and bone tissues.

Methods for treating lysosomal enzymes with the enzymes of the present  
15 invention are within the skill of the artisan. Generally, the lysosomal enzymes is at a concentration of about 10 mg/ml and GlcNAc-phosphotransferase is present in a concentration of about 1 to about 10 million units per milliliter. The enzymes are incubated at about 20°C for about 48 hours or longer in the presence of a buffer that maintains the pH at about 6-7 and any stabilizers or coenzymes required to facilitate the  
20 reaction. Then, phosphodiester  $\alpha$ -GlcNAcase can be added to the system to a concentration of about 250,000 to 100,000 units/mL and the system is allowed to incubate for about 6 or more hours. The modified lysosomal enzymes having highly phosphorylated oligosaccharides is then recovered by conventional means.



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5 In a preferred embodiment, the lysosomal hydrolase at 10 mg/ml is incubated in 50 mM Sodium Acetate pH 6.5, 20 mM MnCl<sub>2</sub>, 0.3 mM (300 μM) with GlcNAc phosphotransferase at 1 to 10 million units/ml at 20°C for 48 hours or longer,. The GBA is then treated with phosphodiester-α GlcNAcase for 6 hours. The modified enzyme is then repurified by conventional chromatography.

10 In a further aspect, the present invention provides a method for the treatment of lysosomal storage diseases by administering a disease treating amount of the highly phosphorylated lysosomal hydrolases of the present invention to a patient suffering from the corresponding lysosomal storage disease. While dosages may vary depending on the disease and the patient, the enzyme is generally administered to the patient in amounts of from about 0.1 to about 1000 milligrams per kg of patient per month, preferably from about 1 to about 500 milligrams per kg of patient per month. The highly phosphorylated enzymes of the present invention are more efficiently taken into the cell and the lysosome than the naturally occurring or less phosphorylated enzymes and are therefore effective  
15 for the treatment of the disease. Within each disease, the severity and the age at which the disease presents may be a function of the amount of residual lysosomal enzyme that exists in the patient. As such, the present method of treating lysosomal storage diseases includes providing the highly phosphorylated lysosomal hydrolases at any or all stages of disease progression.

20 The lysosomal enzyme is administered by any convenient means. For example, the enzyme can be administered in the form of a pharmaceutical composition containing the enzyme and any pharmaceutically acceptable carriers or by means of a delivery system such as a liposome or a controlled release pharmaceutical composition. The term "pharmaceutically acceptable" refers to molecules and compositions that are

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physiologically tolerable and do not typically produce an allergic or similar unwanted reaction such as gastric upset or dizziness when administered. Preferably, "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, preferably humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions, dextrose solutions, glycerol solutions, water and oils emulsions such as those made with oils of petroleum, animal, vegetable, or synthetic origin (peanut oil, soybean oil, mineral oil, or sesame oil). Water, saline solutions, dextrose solutions, and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

The enzyme or the composition can be administered by any standard technique compatible with enzymes or their compositions. For example, the enzyme or composition can be administered parenterally, transdermally, or transmucosally, *e.g.*, orally or nasally. Preferably, the enzyme or composition is administered by intravenous injection.

The following Examples provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention, which is set forth in the appended claims. In the following Examples, all methods described are conventional unless other wise specified.

#### Examples

#### MATERIALS AND METHODS

Construction of the Furin-cleavage site containing  $\alpha/\beta$  subunit of GlcNAc-phosphotransferase - The molecular cloning and expression of wild type human UDP-N-

acetylglucosamine:lysosomal-enzyme-N-acetylglucosamine-1-phosphotransferase  
(GlcNAc-phosphotransferase) is described in U.S. Serial No. 09/636,060 and  
PCT/US00/21970, incorporated herein by reference. Also, the construction and  
expression of recombinant soluble human UDP-N-acetylglucosamine:lysosomal-enzyme-  
5 N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) is described in  
U.S. Serial No. 09/636,060 and PCT/US00/21970, incorporated herein by reference. The  
~~soluble GlcNAc-phosphotransferase  $\alpha/\beta$  subunit cDNA was contained within the Nhe I~~  
and Xba I site of pcDNA 6/V5/His-A (Invitrogen). This plasmid, designated, pMK 52  
was used as the starting material for the construction of the furin-cleavage site containing  
10  $\alpha/\beta$  subunit of recombinant soluble GlcNAc-phosphotransferase as shown in Figure 7.

In order to construct the furin-cleavage site containing  $\alpha/\beta$  subunit of  
recombinant soluble GlcNAc-phosphotransferase, an intermediate plasmid was  
constructed to provide the necessary restriction sites for cloning. The intermediate  
plasmid, designated as pPW6, was constructed by replacing DNA sequence between Hpa  
15 I site to Xba I site with DNA fragments prepared by PCR. This replacement generated  
novel and unique Sfi I and Bsm BI restriction sites which was used to introduce furin  
specific cleavage site between  $\alpha$  and  $\beta$  subunits. Then the sequence between unique Sfi I  
and Bsm BI restriction sites was replaced with synthetic oligo nucleotides which code  
amino acid sequence for furin-cleavage sequence. The plasmid thus made was designated  
20 pPW 9. PPW 9 was used for transient expression of  $\alpha/\beta$  subunit in 293 T cells. PPW9  
was also used as a starting material to make bi-cistronic vectors expressing  $\alpha/\beta$  and  $\gamma$   
subunit of GlcNAc-phosphotransferase(see below). PPW9 was also used to make a  
plasmid for stable expression using pEE14 and pEE14.1 (Lonza Biologics).

Construction of the bi-cistronic expression vector which express Furin-cleavage site containing  $\alpha/\beta$  subunit and wild type  $\gamma$  subunit of GlcNAc-phosphotransferase – To

express both  $\alpha/\beta$  subunit and  $\gamma$  subunit from one vector, the bi-cistronic vector pIRES

5 (Clontech) was used. pIRES has internal ribosome entry sequence between two multiple cloning site, therefore two polypeptides are translated from single mRNA. Nhe I-Xba I fragment of pPW9 which encode furin cleavage site containing  $\alpha/\beta$  subunit was subcloned into Nhe I site of multiple cloning site A of pIRES. Nhe I-Xba I fragment of pMK 17 which encode wild type  $\gamma$  subunit was subcloned into Xba I site of multiple  
10 cloning site B of pIRES. The plasmid thus made was designated pMK 158 and used for transient expression of GlcNAc-phosphotransferase in 293 T cells. To prepare bi-cistronic vector which has pEE14 as a back bone, Nhe I-XbaI fragment of pMK 158 was subcloned into the XbaI site of pEE14 and the plasmid thus made was designated pMK 163.

15 The plasmid pMK 155, expressing  $\alpha/\beta$  was constructed as follows. A *Nhe I-Xba I* fragment from pPW9 was subcloned into the *XbaI* site of pEE14 to prepare a plasmid for stable expression of the  $\alpha/\beta$  GlcNAc-phosphotransferase. The plasmid pMK193 was constructed to express  $\alpha/\beta$  and  $\gamma$  subunits using two promoters rather than the IRES element. The *Nhe I-Xba I* fragment from pPW9 was subcloned into the *XbaI* site of  
20 pEE6.1 (Lonza Biologics) and the cDNA for the  $\gamma$  subunit was subcloned into the *EcoRI* site of pEE 14.1 (Lonza Biologics). These two plasmids were combined together following the manufacturer's instructions to yield a plasmid that expresses  $\alpha/\beta$  and  $\gamma$  subunits from a single plasmid using two CMV promoters.

Transient Expression of Recombinant human GlcNAc-phosphotransferase Enzyme -

The plasmid pMK 158 encoding  $\alpha/\beta$  (furin-cleavage site containing) and subunit of recombinant soluble human GlcNAc-phosphotransferase was initially tested in a

5 mammalian cell culture system using 293T cells for transient protein expression. The transfection of pMK158 into 293T cells was performed using the FUGENE-6 Transfection reagent (Roche) according to the manufacturer's protocol. Briefly, 293T cells were plated in 10-cm dishes at approximately 50% confluency in 10ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal  
10 bovine serum (FBS). Subsequently, 6  $\mu$ g of pMK158 was transfected with 18 $\mu$ l of the FUGENE-6 reagent and the culture was incubated at 37 °C for three days in a 5% CO<sub>2</sub> atmosphere. The media was assayed for GlcNAc-phosphotransferase activity by measuring the transfer of GlcNAc-[<sup>32</sup>P] from the synthetic substrate [<sup>32</sup>P]-UDP-GlcNAc to  $\alpha$ -methylmannoside to produce GlcNAc-[<sup>32</sup>P]monophosphate-methylmannoside. The  
15 medium from the transfected cells was shown to contain active GlcNAc-phosphotransferase and plasmid pMK163 which has same coding sequence with pMK 158 was then used for the stable expression of r GlcNAc-phosphotransferase. Cleavage of the  $\alpha/\beta$  protein *in vivo* was confirmed by SDS-PAGE to compare the size of HPC4 tagged protein, which is the  $\alpha$  subunit.

20

Stable Expression of Recombinant human GlcNAc-phosphotransferase

Stable cell lines were made according to the manufacturer's instructions using the pMK 163, 155 and 193 plasmids described above.

Obviously, numerous modifications and variations on the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as  
5 specifically described herein.

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